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The *lmo0501* gene coding for a putative transcription activator protein in *Listeria monocytogenes* promotes growth under cold, osmotic and acid stress conditions

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Meinem lieben Vater, der
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The *lmo0501* gene coding for a putative transcription activator protein in *Listeria monocytogenes* promotes growth under cold, osmotic and acid stress conditions

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Abstract

In *L. monocytogenes* EGDe, the *lmo0501* gene locus encodes a protein similar to the mannitol transcription regulator (MltR) protein in *B. subtilis* and *B. stearothermophilus*. In this study we investigated its functional role in *L. monocytogenes* EGDe cells in view of growth under different stress conditions. Increased *lmo0501* gene expression at mRNA level was detected in response to cold, osmotic and organic acid stress exposure. An EGDe Δ*lmo0501* mutant strain was diminished in growth compared to the wild type strain in minimal defined medium containing either glucose or fructose, as carbon sources. Furthermore the *lmo0501* null mutant had retarded growth under cold (4°C), salt (NaCl) and organic acid stress conditions compared to the parental wild type strain. Our results confirm the role of the *lmo0501* gene in view of adaptation of *L. monocytogenes* cells to stress conditions as well as a contribution to the efficient utilization of glucose and fructose as carbon sources.

1. Introduction

Listeriosis is a food-borne disease with widely recognized public health and food safety impacts. The invasive form of this disease leads to serious illnesses including septicemia and meningitis, as well as cause high mortality rates in populations at risk, such as old people and immunocompromized individuals. In pregnant women listeriosis can lead to abortions and stillbirths (Posfay-Barbe and Wald, 2009; Ramaswamy et al., 2007; Swaminathan and Gerner-Smidt, 2007). In addition the presence of *Listeria monocytogenes*, the etiological agent of listeriosis, is one of the major problems leading to large scale industrial food product recalls with important economic consequences to food production industries around the world (Kramer et al., 2005).

L. monocytogenes is tough to control due to its ubiquitous occurrence, also in food handling environments, enabling contamination of processed food products. Furthermore this bacterium has a highly adaptable physiology, which facilitates resistance to stress environments that may be imposed through food preservation and hygiene control measures (Gandhi and Chikindas, 2007). In particular major control problems are caused by its psychrotolerance, which enables substantial proliferation on cold preserved ready to eat food products (Chan and Wiedmann, 2009; Tasara and Stephan, 2006). An improved understanding of stress adaptation mechanisms in this bacterium could help to come up with more efficient control measures against this pathogen.

The cold adaptation strategies that have come to light in *L. monocytogenes* include molecular response mechanisms designed to maintain flexible functional cell membrane states, as well as to increase the uptake of cryoprotective nutrients in response to low temperature stress (Angelidis and Smith, 2003; Zhu et al., 2005). Additional molecular cold protection systems have been described but an improved understanding of most of these systems is still required. Such mechanisms include general stress response proteins

such as low temperature control proteins (LtrC), cold shock domain family proteins (Csps), alternative sigma factors, two component regulatory proteins, ferritin and catalase, as well as UD-glucose phosphorylase Lmo1078, a putative RNA helicase and the PgpH protein (Azizoglu and Kathariou, 2010a; Azizoglu and Kathariou, 2010b; Becker et al., 2000; Chan et al., 2008; Chassaing and Auvray, 2007; Dussurget et al., 2005; Liu et al., 2006; Schmid et al., 2009; Zheng and Kathariou, 1994).

In addition several other putative cold, osmotic, acid and alkaline stress protection genes and proteins are known from gene expression analysis, which now allows rationalized creation of deletion mutants for functional studies (Bowman et al., 2010; Chan et al., 2007; Giotis et al., 2008; Giotis et al., 2010; Liu et al., 2002). One of the putative cold adaptation genes identified in this bacterium is the *lmo0501* locus of *L. monocytogenes* EGDe, which codes for the putative transcription activator protein Lmo0501. An increase in *lmo0501* mRNA is observed in transcriptomes of cold acclimated *L. monocytogenes* cells (Chan et al., 2007; Liu et al., 2002). The aim of the present study was to examine *lmo0501* gene functional contributions in adaptation of *L. monocytogenes* cells to cold, NaCl salt and organic acid stress associated with preserved food.

2. Material and Methods

2.1. Culture media and bacterial strains

Strains were grown in brain-heart infusion (BHI pH 7.4; Oxoid, Hampshire, UK) or defined minimal media (DM; (Premaratne et al., 1991), which contained glucose (1% or 10%) or fructose (1%). DMS was prepared by supplementing DM with 4% of NaCl. BHI was acidified by addition of 2.5% lactic acid or 0.35% acetic acid and pH adjustment to 6.0 (BHI-lactic acid) and 5.5 (BHI-acetic acid), using NaOH. The acidified BHI broths

were stabilized by inclusion of 9.8g/L MES-hydrate (Sigma Aldrich, Switzerland). The *L. monocytogenes* EGDe wild type and EGDe Δ*lmo0501* strains were used. The EGDe Δ*lmo0501* strain was constructed by in frame deletion of the *lmo0501* gene locus. A deleted copy of this gene, in which the reading frame and the first six and last ten codons were retained, was created using the splicing-by-overlap extension (SOE) PCR and primers listed in table 1 as previously described (Horton et al., 1990). The EGDe Δ*lmo0501* DNA fragment was cloned into the temperature sensitive *pKSV7* plasmid (Smith and Youngman, 1992), which was subsequently used in replacement of the EGDe chromosomal copy of *lmo0501* based on a series of homologous recombination events (Schmid et al., 2009). PCR analysis and DNA sequencing confirmed the *lmo0501* locus deleted mutant. An *E. coli* XL1-Blue host strain was used for the different plasmid molecular manipulations as previously described (Raimann et al., 2010).

2.2. Growth conditions

The EGDe wild type and Δ*lmo0501* strains were grown (37°C, 150 rpm) to the stationary phase after inoculation of 10 ml BHI cultures with single colonies. These stationary phase cells were diluted to approximately 10^3 CFU/ml in 10 ml of: (i) BHI (pH7.4), BHI-lactic acid (pH 6.0), BHI-acetic acid (pH 5.5), DM and DMS. Samples were incubated at 37, 10 and 4°C. Growth was monitored by standard colony counting methods. The lag phases and growth rates ($\log_{10}$ (CFU/ml)/hr) of the strains were estimated from log converted colony counts (CFU/ml) using the Dmfit program (version 2.0), which is based on the Baranyi model (Baranyi and Roberts, 1994).

2.3. Stress exposure, total RNA extraction and cDNA synthesis

Stationary phase EGDe cells prepared as described above were diluted to 10^8 CFU/ml (OD_{600} 0.01) in 100 ml of: (i) BHI (pH 7.4), (ii) BHI-lactic acid (pH 6.0), (iii) DM and (iv) DMS. These cultures were incubated at 37 and 10°C and their growth were

monitored based on colony counting. One and half milliliters of mid exponential growth phase cultures were collected. RNA profiles in samples were immediately preserved using the bacterial RNA Protect Reagent (Qiagen). Total RNA was extracted as previously described (Arguedas-Villa et al., 2010). Samples were controlled for potential residual DNA contamination prior to reverse transcription by amplification. Ten nanograms total RNA from each sample was amplified in real-time qPCR using *16S rRNA* and *lmo0501* specific PCR primers and previously described PCR cycling conditions (Arguedas-Villa et al., 2010). As a positive control, similar amounts of EGDe genomic DNA templates were also amplified. RNA samples giving no amplification products under these conditions were considered free of DNA contamination. Six hundred nanograms of such DNA free total RNA templates were converted into cDNA using the Quantitect Reverse Transcription kit (Qiagen).

2.4. Relative quantification of lmo0501 gene expression

Real-time PCR amplification was performed in the Light Cycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland) using primers listed in table 1. Previously described amplification conditions were used and the *16S rRNA* and *lmo0501* gene specific primers were optimized to achieve specific target amplification ranging from 95 to 100% PCR efficiency (Arguedas-Villa et al., 2010). 7.2 ng of cDNA prepared as described above were used as templates in qPCR assays. These were amplified in ten microlitre reaction mixtures containing primers (400nM) and LightCycler^{R} 480 SYBR Green I master mix (Roche molecular Diagnostics, Penzburg, Germany). A calibrator based on total RNA isolated from stationary phase EGDe cells grown as described above was applied. Reference gene validation was performed as previously described (Pfaffl et al., 2004; Tasara and Stephan, 2007) and this process confirmed *16S rRNA* to be suitable as an internal control reference gene under the different experimental conditions used in

this study. The *lmo0501* transcripts were therefore quantified relative to *16S rRNA* transcript levels in the same sample and normalized to the calibrator using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics).

2.5. Statistical analysis

The statistical analysis was carried out using the JMP statistical software package (version 8.0; SAS Institute, Cary, NC). Statistical significance of growth differences between the EGDe wild type and Δ*lm0501* strains were determined using the one-way analysis of variance (ANOVA) test. Gene expression ratios were first normalized through $\log_{10}$ conversion before analysis with the Students t-test to determine the statistical significance of gene expression differences between controls and stress exposed EGDe cells. In all cases differences with P values <0.05 were considered as statistically significant.

3. Results

3.1. The lmo0501 *gene expression in L. monocytogenes* EGDe *cells is induced by exposure to cold, organic acid and NaCl osmotic stress.*

Quantitative real-time reverse transcription PCR (qRT-PCR) was used to assess the *lmo0501* gene expression responses to cold, osmotic and organic acid stress exposure. The *lmo0501* mRNA levels were significantly induced ($P<0.05$) in *L. monocytogenes* EGDe cells exposed to all of the three different stress conditions examined. On average *lmo0501* mRNA levels were two and half-fold higher in cold than 37°C temperature grown *L. monocytogenes* EGDe cells in BHI (Fig. 1A). Similarly up to five-fold more *lmo0501* mRNA induction was detected in lactic acid stress exposed EGDe cells in BHI at 37°C (Fig. 1A). NaCl osmotic stress exposure of EGDe cells at 37°C had no impact on

lmo0501 gene expression levels, but it also induced the expression of this gene at 10°C (Fig. 1B). The *lmo0501* transcripts were thus on average four-fold higher in EGDe cells grown in DMS (DM plus 4% NaCl) than DM at 10°C.

*3.2. Growth of the EGDe Δ*lmo0501 *mutant on fructose and glucose*

Further examination of the two strains in defined minimal media broth revealed slower growth rates of EGDe *Δlmo0501* compared to the wild type strain in presence of fructose (0.109±0.007 vs 0.221±0.039 log/hr) and glucose (0.154±0.013 vs 0.215±0.040 log/hr) as carbon sources at 1% (Fig. 2A and 2B). In contrast no phenotypic growth differences were found in the *lmo0501* mutant in DM when glucose levels were increased to 10% in this media (Figs. 2C). Based on these observations, 10% glucose level in DM was adopted for all further experimental analysis of these two strains.

*3.3. Growth of the EGDe wild type and EGDe Δ*lmo0501 *strains exposed to cold, osmotic and organic acid stress*

The cold growth phenotypes of the EGDe wild type and Δ*lmo0501* strains were examined in DM and BHI cultures. No growth differences were observed between the two strains in BHI at 10 and 4°C (data not shown), as well as in DM exposed to mild cold stress at 10°C (Fig. 3A). On the other hand in DM at 4°C, the Δ*lmo0501* mutant strain displayed prolonged lag phase period (3.83±1.2 vs 2.12±0.72 weeks), and slower exponential growth rate (0.0030±0.0006 vs 0.0046±0.0005) than the wild type strain (Fig. 3B). In addition, the cell counts achieved by the mutant after eight weeks of incubation were lower (5.23±0.72 vs 7.05±0.26 log CFU/ml) compared to the wild type strain. We next compared the growth of the two strains exposed to NaCl osmotic stress in both BHI and DM cultures at 37°C. No phenotypic growth differences were detected between the two strains in BHI plus 4% NaCl (data not shown). In contrast, the Δ*lmo0501* strain had significantly ($P<0.05$) retarded growth in DM plus 4% NaCl compared to the wild type

(Fig. 3C). It was characterized by a prolonged lag phase (55.3±9.0 vs 25.22±11.98 hrs), and slower growth rate (0.037±0.013 vs 0.015±0.010 log/hr) relative to the wild type.

The contribution of the *lmo0501* function to *L. monocytogenes* adaptation to organic stress conditions was also examined. Despite similar growth to the wild type strain in normal BHI, the EGDe *Δlmo0501* strain also grew significantly ($P<0.05$) slower under lactic and acetic acid stresses in BHI at 4°C (Figs. 4A-C). The EGDe *Δlmo0501* strain displayed prolonged lag phases (3.84±0.12 vs 5.5±0.01 weeks), but similar exponential growth rates (0.0060±0.00006 vs 0.0057 ±0.00024 log/week), to the wild type under lactic acid stress in BHI (Fig. 4B). While this mutant failed to grow during the time course of the experiment (twelve weeks), the wild type strain increased by about one and half logs in presence of acetic acid stress at pH 5.5 (Fig. 4C).

4. Discussion

The *lmo0501* gene locus is part of a multigene operon cluster (*lmo0498-0508*) predicted in the EGDe genome to include several genes linked to carbon assimilation and metabolism functions (Glaser et al., 2001). The Lmo0501 protein is predicted to be a 686 amino acid long transcription regulator, with a topological domain organization (Fig. 5), which resembles the mannitol transcriptional activators (MtlR) in *B. subtilis* and *B. stearothermophilus* (Henstra et al., 2000; Henstra et al., 1999; Watanabe et al., 2003). In addition to conserved DNA binding winged helix –turn- helix (HTH), MtlRs possess two PTS regulation domains, PRDI and PRDII, that are phosphorylation regulated by PTS components (van Tilbeurgh and Declerck, 2001). Beside these three domains, the Lmo0501 protein also possesses PTS enzyme II motifs that resemble the $EIIB^{bgl\text{-}like}$ and $EIIA^{fructose/mannitol}$ subunits. While *B. subtilis* and *B. stearothermophilus* MtlRs are known

transcription regulators activating the expression of mannitol assimilation and catabolism genes, the Lmo0501 functions in *L. monocytogenes* are presently unknown (Henstra et al., 2000; Henstra et al., 1999; Watanabe et al., 2003).

In agreement with previous reports (Chan et al., 2007; Liu et al., 2002), cold stress dependent *lmo0501* mRNA induction could also be detected in the present study. In addition an *lmo0501* transcriptional induction was detected in response to lactic acid and NaCl osmotic stresses. Furthermore *lmo0501* gene deletion impaired the growth of *L. monocytogenes* EGDe cells exposed to cold (DM 4°C), osmotic (DM plus 4% NaCl) and organic acid (BH-lactic (pH 6.0) and BHI-acetic acid (pH 5.5)) stress conditions. In addition our findings also suggest that the *lmo0501* gene locus might also influence the growth of *L. monocytogenes* cells on glucose and fructose carbon sources.

The *lmo0501* gene is found within an operon, which includes carbon metabolism enzymes (ribulose 5-phosphate isomerase, ribulose-5-phosphate 3 epimerase, transaldolase, sugar-phosphate isomerase and polyol (sorbitol) dehydrogenase) and PTS components ($EIIA^{fruc}$, $EIIB^{gal}$ and $EIIC^{gal}$) involved in specific carbon source assimilation (Glaser et al., 2001; Stoll and Goebel, 2010). Assuming that Lmo0501 is a transcriptional activator in *L. monocytogenes*, it is plausible that one of its roles might include transcriptional activation of this operon. Such a function would enhance cellular energy generation capacity in normal grown as well as stress challenged *L. monocytogenes* organisms. As such reduced $EIIA^{fruc}$, $EIIB^{gal}$ and $EIIC^{gal}$ production would be expected in *lmo0501* deleted *L. monocytogenes* cells leading to reduced ability to optimally utilize certain carbon sources. This might thus explain reduced growth rates of the EGDe Δ*lmo0501*mutant on glucose or fructose in DM as observed in our study. Along the same lines cells of this bacterium exposed to cold, osmotic and organic acid stress also face increased metabolic and energy demands to deal with various molecular and

physiological stress challenges. The EGDe Δ*lmo0501* mutant cells facing with such challenges might fail to efficiently resolve the associated physiological demands, since they would also be impaired in assimilation and metabolism of some carbon sources from the growth environment. These defects would explain the slower growth recovery and growth rates observed in *lmo0501* null cells exposed to these stress conditions as observed in our study.

In summary our study supports the functional contribution of *lmo0501* to physiological processes, which promotes adaptation of *L. monocytogenes* cells to cold, osmotic and organic acid stress conditions. In terms of its putative functions this gene codes for a putative MtlR-like transcription activator, which based on knowledge from other bacteria, might activate the expression of genes associated with assimilation and metabolism of some carbon sources. Even though the targeted genes and mechanisms of *lmo0501* dependent gene expression regulation are yet to be delineated in *L. monocytogenes*, our phenotypic findings presented here suggest that, some of the *lmo0501* regulated target genes in this bacterium may be important in glucose and fructose assimilation and metabolism.

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TABLES

TABLE 1. Primers[1] used in this study

Primers	Sequence (5'→ 3')
SOE-P1-lmo0501A[2]	TCCCCCGGGGACAGTTGGTTTTACGTATGAGGA
SOE-P2- lmo0501B[3]	*GATTGTTTGCATGATTCTGTTATCCAGATA*CATGCGTCT
SOE-P3- lmo0501C[3]	*TATCTGGATAACAGAATCATGCAAACAATC*AACCAATTAGTA
SOE-P4- lmo0501D[2]	TCCCCCGGGCCTCCGACTGGCTGCT
lmo0501-fw[4]	GAATCAGAGATCGGCG
lmoo501-rv	CGGTAATAGGCTGGTT
16S rRNA-fw	CTTCCGCAATGGACGAAAGT
16S rRNA-rv	CTCATCGTTTACGGCGTG

[1]Oligonucleotides were synthesized at Microsynth AG (Balgach, Switzerland);

[2]The *Sma*I restriction sites incorporated to facilitate cloning are underlined

[3]The complimentary overlapping regions of SOE-P2-lmo0501B and SOE-P3-lmo0501C are in italics

[4]Quantitative real-time PCR primers were designed using the LC probe and primer design software (Roche Molecular Diagnostics GmBH, Penzburg, Germany)

Figure legends

Fig. 1. Relative quantification of *lmo0501* mRNA levels in mid exponential EGDe cells grown exposed to: (A) cold (10°C) and acid (BHI plus 2.5 % lactic acid pH 6.0) stress, and (B) osmotic (DM plus 4% NaCl) stress. The means and standard deviations (±1SD) of three independent experiments are presented. The letters above the bars denote statistically significant differences compared to the controls ($P<0.05$).

Fig. 2. Growth of the EGDe wild type and *Δlmo0501* strains in DM containing glucose, fructose, and sucrose. The two strains were inoculated in DM containing (A) 1% glucose, (B) 1% fructose, (C) 10% glucose. The means and standard deviations (±1SD) of three independent experiments are presented.

Fig. 3. Growth of the EGDe wild type and *Δlmo0501* strains exposed to cold and NaCl stress. The two strains were inoculated in DM and incubated at (A) 10°C and (B) 4°C, as well as in DMS (C) and incubated at 37°C. The means and standard deviations (±1SD) of three independent experiments are presented.

Fig. 4. Growth of the EGDe wild type and *Δlmo0501* strains exposed to organic acid stress. The two strains were inoculated in (A) BHI (pH 7.4), (B) BHI plus 2.5% lactic acid (pH 6.0) and (C) BHI plus 0.35% acetic acid (pH 5.5). The means and standard deviations (±1SD) of three independent experiments are presented.

Fig. 5. The gene cluster around the *lmo0501* gene in the *L. monocytogenes* EGDe genome and the topological domain arrangement of the *L. monocytogenes* EGDe Lm0501 and the MtlRs of *B. subtilis* and *B. stearothermophilus* are shown.

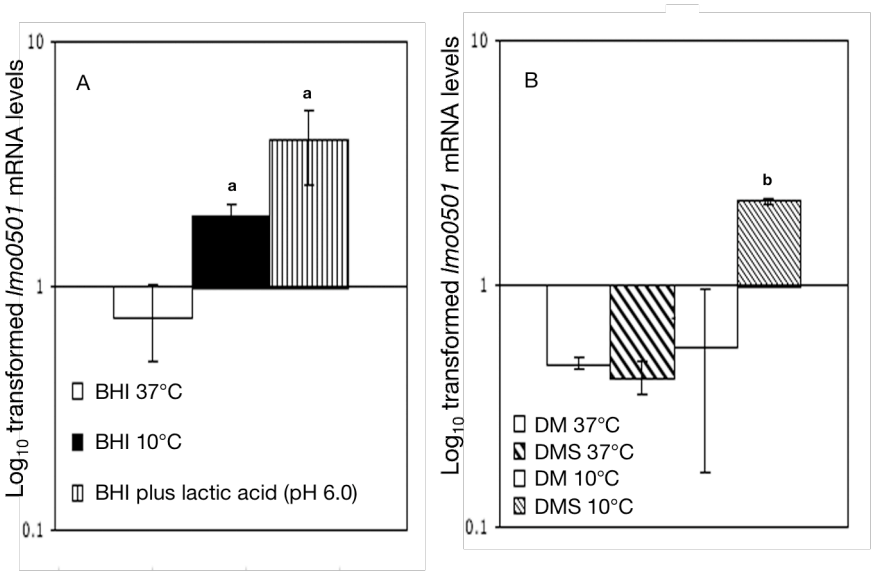
A
B
10
1
0.1
a
a
b
Log10 transformed lmo0501 mRNA levels
BHI 37°C
BHI 10°C
BHI plus lactic acid (pH 6.0)
DM 37°C
DMS 37°C
DM 10°C
DMS 10°C

Figure 1

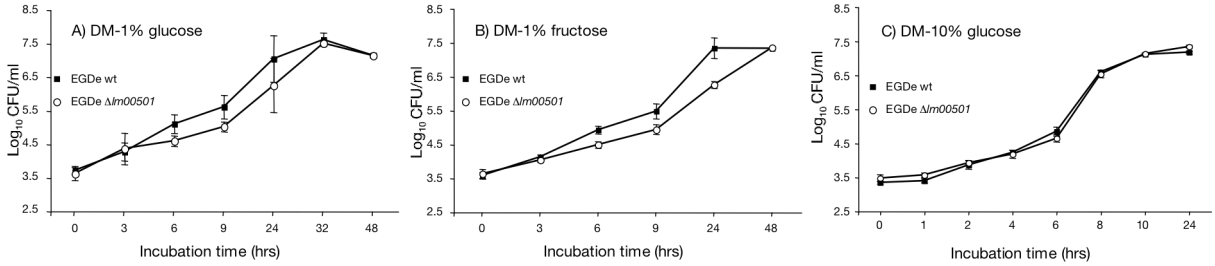
A) DM-1% glucose
B) DM-1% fructose
C) DM-10% glucose
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (hrs)
8.5
7.5
6.5
5.5
4.5
3.5
2.5
0
3
6
9
24
32
48
1
2
4
8
10

Figure 2

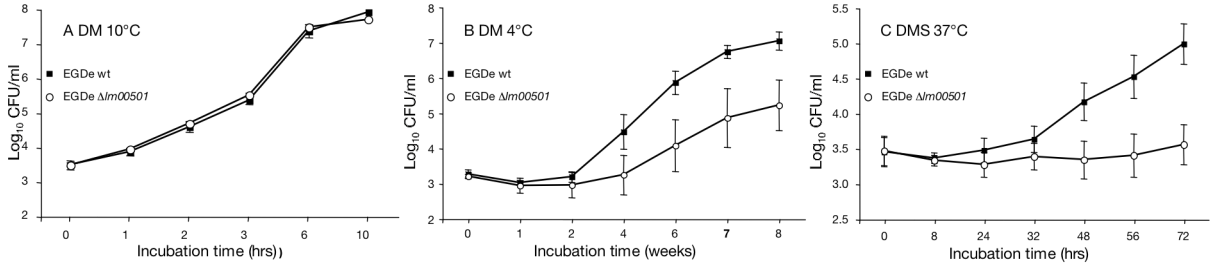
A DM 10°C
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (hrs)
B DM 4°C
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (weeks)
C DMS 37°C
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (hrs)

Figure 3

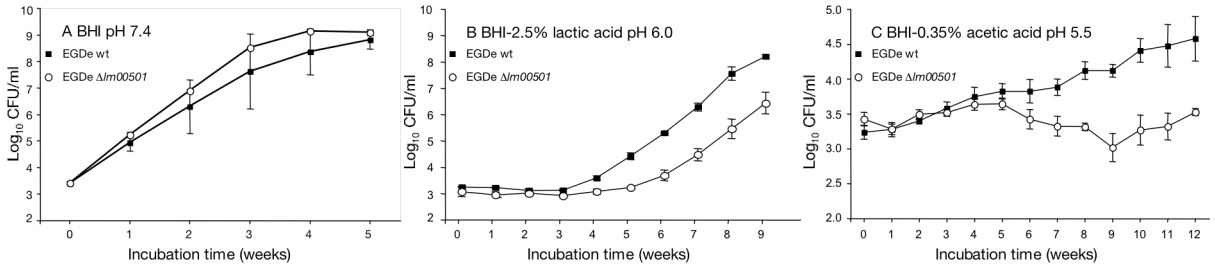
A BHI pH 7.4
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (weeks)
B BHI-2.5% lactic acid pH 6.0
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (weeks)
C BHI-0.35% acetic acid pH 5.5
EGDe wt
EGDe Δlm00501
Log10 CFU/ml
Incubation time (weeks)

Figure 4

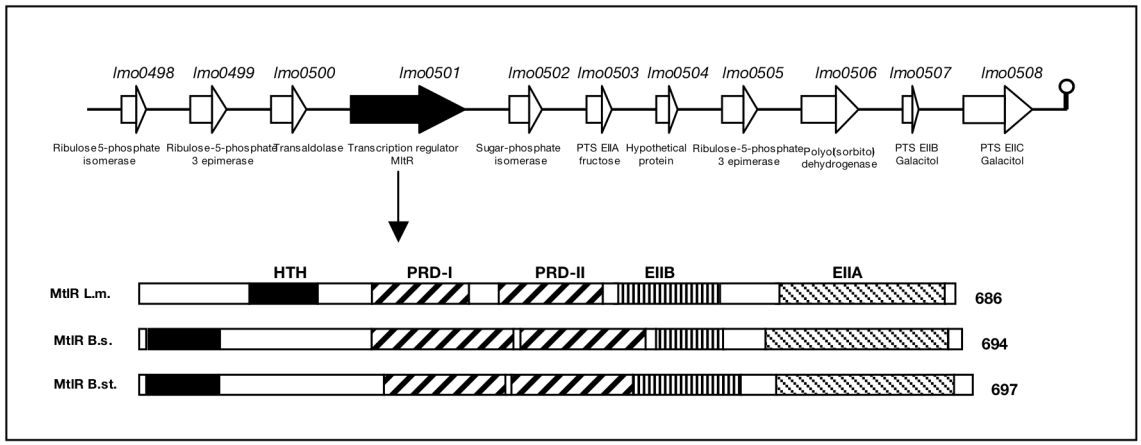
lmo0498
lmo0499
lmo0500
lmo0501
lmo0502
lmo0503
lmo0504
lmo0505
lmo0506
lmo0507
lmo0508
Ribulose5-phosphate isomerase
Ribulose-5-phosphate 3 epimerase
Transaldolase
Transcription regulator MltR
Sugar-phosphate isomerase
PTS EIIA fructose
Hypothetical protein
Ribulose-5-phosphate 3 epimerase
Polyol(sorbito) dehydrogenase
PTS EIIB Galacitol
PTS EIIC Galacitol
HTH
PRD-I
PRD-II
EIIB
EIIA
MtlR L.m.
686
MtlR B.s.
694
MtlR B.st.
697

Figure 5

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